

Light-emitting Diode Phototherapy at 630 ± 3 nm Increases Local Levels of Skin-homing T-cells in Human Subjects

Shin-ichiro Takezaki¹, Tokuya Omi^{1,2}, Shigeru Sato³ and Seiji Kawana¹

¹Department of Dermatology, Nippon Medical School

²Department of Dermatology, Queen's Square Medical Center

³Central Institute for Electron Microscopic Research, Nippon Medical School

Abstract

Background and aims: Red light phototherapy with laser sources has been used successfully for a number of indications. A new generation of quasimonochromatic 630 ± 3 nm light-emitting diode (LED) systems has recently been yielding good results for the same indications, but no study has examined changes in visible red light irradiated skin at an immunological level. This study was thus designed to examine changes in skin-homing T-cell levels induced in normal human skin by visible red LED energy.

Subjects and methods: Six adult male volunteers (35~48 years old) who satisfied all study criteria had the skin over the lateral aspect of the leg irradiated once per week for 8 weeks with a visible red (630 ± 3 nm) LED-based system, with irradiance of 105 m/cm^2 , 15 minutes/session, and a radiant flux of 94 J/cm^2 . Skin biopsies were performed after the eighth treatment session, and cultures were prepared to assay the type and quantity of skin-homing T-cells using qualitative and quantitative polymerase chain reaction (PCR) techniques. Ultrastructural changes were also assessed with transmission electron microscopy.

Results: Transmission electron microscopy revealed mild fibroplastic changes in fibroblasts, with no acute inflammatory changes throughout the treatment session. Qualitative PCR showed the presence of both Th-1 and Th-2 T-cells, and quantitative PCR showed an increase in the numbers of both types of skin-homing T-cells, much more so for Th-2 than for Th-1.

Conclusions: Visible red LED irradiation appears to activate the skin-homing immune system.

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Key words: light-emitting diode phototherapy, skin-homing T-cells, Th-1 cell, Th-2 cell

Introduction

The morphological effects of visible red light therapy with laser sources delivering low incident

levels of photons, known as low reactive-level laser therapy, low incident laser irradiation, and other such terms, have been well documented in the literature. Recent studies in soft tissue and bone¹⁻³, and a meta-analysis⁴ have shown that visible red

Correspondence to Seiji Kawana, MD, Department of Dermatology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan

E-mail: kawa2559@nms.ac.jp

Journal Website (<http://www.nms.ac.jp/jnms/>)

light has significant effects in accelerating wound healing in hard and soft tissues and is effective in the control of inflammation⁵.

With recent developments in light-emitting diode (LED) technology, LEDs are now available with laser-like, quasi-monochromatic output, having bandwidths as low as ± 3 nm from the rated wavelength. When mounted in arrays, this new generation of LEDs can deliver clinically useful incident energy densities from hand-free-type systems that cost less than laser systems. One such system, the Omnilux Revive (Photo Therapeutics, Ltd, Altrincham, UK), was granted 510-K Food and Drug Administration approval in July 2003 for general dermatological applications. Beneficial effects of LED technology have been reported at the morphological and molecular levels⁶⁻⁸, but to our knowledge no report has yet appeared on the immunological effects of red light therapy with LEDs.

Among the many immunological components that are driven in the skin, skin-homing T-cells are known to play the most crucial roles, which are ① organ-specific trafficking into the skin, ② signal transmission from dendritic cells, and ③ induction and regulation of many inflammatory process in the skin⁹.

In our previous study, we have observed increased levels of interleukin (IL)-2 and IL-4 messenger (m) RNA in skin-homing T lymphocytes after treatment with the low fluence pulsed dye laser¹⁰. Thus, in the present study, we analyzed the effects of a newly developed LED phototherapy at 630 ± 3 nm on the changes in skin-homing T-cell levels in normal human skin.

Subjects and Methods

Therapy Source

Phototherapy was delivered with a red LED-based free-standing unit (Omnilux Revive, Photo Therapeutics Ltd.). The treatment head is composed of an array of visible red LEDs ($\lambda = 630 \pm 3$ nm, irradiance of 105 m/cm^2 , 15 min/session, radiant flux of approximately 94 J/cm^2 , active area of approximately 22×18 cm) precisely mounted in 4

movable panels that enable the treatment head to follow the contour of the area to be treated, in this case the lateral aspect of the leg. The head is attached to an articulated arm and was set up on the base unit control console, and, when the subject had donned appropriate protective eyewear, light therapy was started.

Trial Subjects

Six healthy adult volunteers (mean age, 31.7 years; range, 35~48 years) were recruited for this study, and informed consent was obtained from all subjects.

Exclusion criteria included chronic illness, cutaneous contact dermatitis, atopic dermatitis, photosensitivity, history of scar formation, vascular disease, stasis dermatitis, history of poor wound healing, and any form of cutaneous disease. All subjects were Japanese and had Fitzpatrick's type IV skin. The study was approved by the Ethics Committee of the Japan Equestrian Federation for Sports Medicine Research.

Procedure

In each subject, an area was chosen on the lateral aspect of the leg over the fibula. Each area was treated with the Omnilux Revive at the above parameters once per week for 8 weeks.

Ultrastructural Analysis

Three-millimeter skin punch biopsies were obtained from each subject after the second and eighth treatment sessions. The specimens were fixed with 2.5% glutaraldehyde and postfixed with 1% osmium tetroxide. The tissue samples were dehydrated through a graded alcohol series and embedded in Epon 812. Ultrathin sections were cut using an Ultracut N Ultramicrotome (Reichert-Nissei, Tokyo) with a diamond knife and were stained with uranyl acetate and lead citrate. The sections were examined under a transmission electron microscope (TEM; 75kV, Hitachi H-7500; Hitachi, Tokyo, Japan).

Skin-homing T-cell Culture

Skin-homing T-cells were cultured with the well-established method of Kaltoft et al.¹¹⁻¹³. In this

Effects of 630±3 nm Light on Normal Human Skin

Table 1 Bands for the identification of Th-1 (IL-2 products) and Th-2 (IL-4 products) skin-homing T-cells following RT-PCR

| restriction enzyme | Expected bands (bp) | |
|--------------------|---------------------|------------------|
| | IL-2 mRNA (Th-1) | IL-4 mRNA (Th-2) |
| Alu I | 184, 274 | 306, 153 |
| Hae III | 250, 208 | 347, 112 |

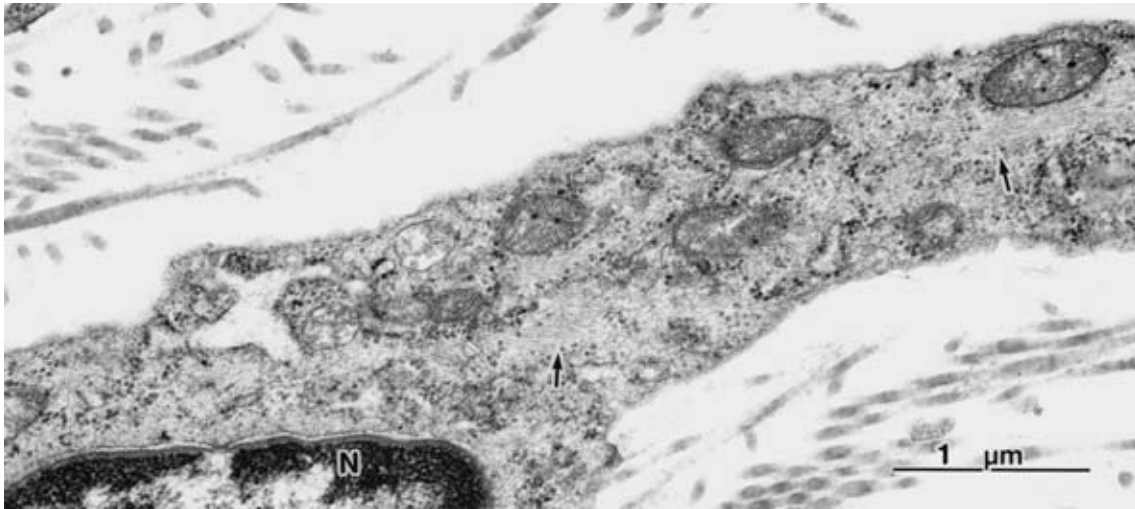


Fig. 1 Transmission electron micrograph of part of a fibroblast after the 2nd irradiation (2 weeks into the study). Mild interstitial and perivascular edema can be seen, and vimentin fibers (**arrows**) are conspicuous in the fibroblast cytosol, with an increased number of mitochondria (bar=1 μ m). N: Nucleus

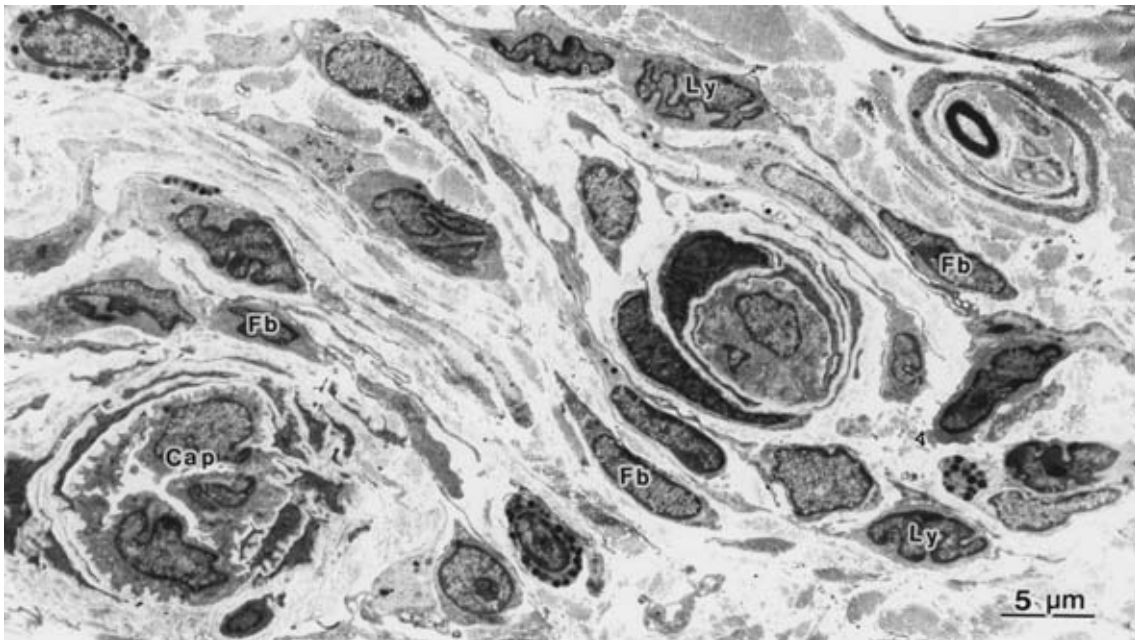


Fig. 2 Transmission electron micrograph of 630 \pm 3 nm-irradiated tissue after the eighth treatment session, 8 weeks into the study. An increase in the number of fibroblasts (Fb) with the migration of lymphocytes (Ly) is observed (bar=5 μ m). Cap: Capillary

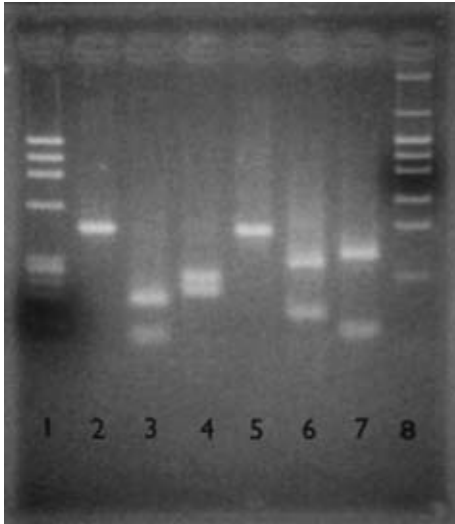


Fig. 3 PCR products are visualized on an ethidium bromide-stained agarose gel. Bands were detected where expected, with IL-2 amplification products associated with Th-1 skin-homing T cells seen at the 93-, 181-, and 184-bp bands (Alu I) and at the 208- and 250-bp bands (Hae III). IL-4 amplification products for Alu I and Hae III, associated with Th-2 skin-homing cells, appear at bands of 306 and 153 bp and of 347 and 112 bp, respectively.

Lane 1: Hae III marker (0.02 µg/µl × 10 µl)

Lane 2: IL-2 amplification products (458 bp)

Lane 3: IL-2 amplification products (93, 181, and 184 bp) (Alu I)

Lane 4: IL-2 amplification products (208, 250 bp) (Hae III)

Lane 5: IL-4 amplification products (459 bp)

Lane 6: IL-4 amplification products (306, 153 bp) (Alu I)

Lane 7: IL-4 amplification products (347, 112 bp) (Hae III)

Lane 8: PHY marker (0.02 µg/µl × 10 µl)

method, T cells in a skin biopsy can proliferate by using T cell growth factors (IL-2 and IL-4).

Three-millimeter skin punch biopsies were obtained from each subject after the eighth treatment session. Each biopsy specimen was cultured for 6 weeks at 37°C in 8-ml Roswell Park Memorial Institute (RPMI) 1,640 medium containing 10% fetal bovine serum (FBS), 100 U/ml IL-2, 250 U/

ml IL-4, 100 U/ml penicillin, and 100 µg/ml streptomycin.

Isolation of RNA from Cultured T-cells

T cells (at least 1,000,000 per culture specimen) were harvested by draining the culture media into test tubes and centrifuging at 1,500 rpm for 5 minutes. The pellet was washed by resuspending it in phosphate-buffered saline and centrifuging at 1,500 rpm for 5 minutes. This process was repeated twice. RNA was extracted by adding 1.3 ml of RNAzol B (Tel-Test, Inc., Friendswood, TX, USA) and 130 µl of chloroform to the final pellet and centrifuging the suspension at 15,000 rpm for 20 minutes at 4°C. The pellet was then subjected to consecutive rounds of isopropyl alcohol and ethanol precipitation and dissolved in distilled water.

Qualitative Reverse Transcriptase Polymerase Chain Reaction

One microgram of total RNA isolated from each sample was subjected to reverse transcription using a 100-µg mixture of random hexamers, 1 mM of each deoxynucleotide triphosphate (d-NTP) (Takara, Tokyo, Japan), and 2 U/µl M-MLV reverse transcriptase (Invitrogen, Carlsbad, California, USA), in reverse transcription buffer (3 mM MgCl₂, 75 mM KCl, 50 mM Tris-HCl, pH 8.3). The mixture was allowed to react at 60°C for 5 minutes before rapidly cooling to 4°C. The reaction was stopped by the addition of 100 mM DTT, 20 U reverse transcriptase (RT), and 1.0 to 1.5 U/µl RNasin (Takara, Tokyo, Japan), and incubated successively at 37°C for 60 minutes and 95°C for 5 minutes, before being allowed to sit at 4°C.

The resulting complimentary (c)DNA was amplified by the polymerase chain reaction (PCR) technique using a thermal cycler (Astec, Fukuoka, Japan). The actual reaction mixture consisted of 5 pmol each of the forward and reverse oligonucleotide primers, and 1.25 U recombinant Taq DNA polymerase (Amersham-Pharmacia Biotech, Piscataway, NJ, USA) in 50 µl PCR buffer (2 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, pH 8.3). The PCR primers for IL-2 cDNA and IL-4 cDNA were synthesized on the basis of published sequence

Table 2 Semiquantitative RT-PCR analysis of cultured skin-homing T-cells, comparing copies per µg of IL-2 (Th-1 T cells) and IL-4 Th-2 T-cells) mRNA

| Subject | Copy/µg RNA | | β-actin |
|---------|-------------|--------|---------|
| | IL-2 | IL-4 | |
| T.H. | 17 | 7,000 | + |
| Y.H. | 42 | 15,000 | + |
| H.K. | 11 | 13,000 | + |
| K.O. | 18 | 9,000 | + |
| H.S. | 22 | 7,000 | + |
| S.K. | 150 | 8,400 | + |

data¹⁴. The PCR amplification itself was performed for 36 cycles, each cycle consisting of a 30-second denaturation at 94°C, a 1-minute renaturation at 55°C, and a 2-minute incubation at 74°C. Following the completion of the final cycle, all samples were incubated for an additional 3 minutes at 74°C.

Identification of PCR Products

The PCR products were identified by digesting the samples with restriction enzymes Alu I and Hae III, electrophoresing on a 3% (w/v) agarose gel, and staining with ethidium bromide. The presence of IL-2 amplification products would result in bands of 93 bp, 181 bp, and 184 bp bands with Alu I, and bands of 208 bp and 250 bp with Hae III. The presence of IL-4 products would result in 306-bp and 153-bp bands with Alu I and 347-bp and 112-bp bands with Hae III (**Table 1**).

Quantitative RT-PCR

The basic procedure was the same as for qualitative PCR except that a total of eight predetermined external standards ranging from 10 to 1,000,000 copies of DNA were included with each amplification run. The undigested products were electrophoresed on a 3% (w/v) agarose gel, stained with ethidium bromide, and photographed. The number of copies of IL-2 and IL-4 mRNA in the original lymphocyte cultures were calculated by comparing the intensities of ethidium bromide-stained bands of the test samples with those of the predetermined standards. This was accomplished by quantifying the various band intensities with a reflectance densitometer as they appeared on the photograph of the gel. β-actin was used as a control

for the PCR reaction.

Results

Ultrastructural Analysis

After the second treatment session TEM revealed mild interstitial edema and increased vimentin fibers that could be identified particularly in the fibroblasts (**Fig. 1**). After the eighth treatment session increased fibroblast count was increased (**Fig. 2**), and vimentin granules and an increased number of mitochondria could be clearly observed in the fibroblasts. No acute inflammatory changes were observed in the specimens throughout the treatment session.

Skin-homing T-cell Analysis

Qualitative RT-PCR: **Figure 3** shows PCR products as visualized on an ethidium bromide-stained agarose gel. Bands were detected where expected, with IL-2 amplification products associated with Th-1 skin-homing T-cells seen at the 93-, 181-, and 184-bp bands (Alu I) and at the 208- and 250-bp bands (Hae III). IL-4 amplification products for Alu I and Hae III, associated with Th-2 skin-homing T-cells, appear as bands of 306 and 153 bp, and 347 and 112 bp, respectively.

Quantitative RT-PCR: As seen in **Table 2**, increases in the numbers of both types of cultured skin-homing T-cells could be deduced on the basis of the amplification results of IL-2 and IL-4 RNA. In particular, the large copy numbers registered for IL-4 mRNA indicate a larger increase in the number of Th-2 T-cells when compared with that of Th-1 T-cells.

Discussion

Most immunological studies have focused on antigen-specific T lymphocytes obtained from peripheral blood^{15,16}. On the other hand, 0.5 to 1.5×10^6 skin-homing T lymphocytes are present in a 4-mm punch biopsy of atopic dermatitis skin, and these T lymphocytes play important roles in the cutaneous immune system¹⁷.

Kaltoft *et al*^{11,12} have established a culture technique for skin-homing T lymphocytes from patients with atopic dermatitis using IL-2 and IL-4. These T-cells are CD4(+) and lose T-cell antigen receptor complex expression after prolonged culture. Kaltoft *et al* have performed several studies of allergic skin diseases using these skin-homing T-lymphocyte system^{11,13,17,18}.

Several studies of immunological changes after phototherapy have been published^{19,20}. In our previous study, we observed increased levels of IL-2 and IL-4 mRNA in skin-homing T lymphocytes after treatment with the low fluence pulsed dye laser¹⁰. For these reasons, we decided to evaluate the effects of a newly developed 630 ± 3 nm LED phototherapy on the skin-homing T lymphocyte system.

In this study, we investigated the immunological characteristics of cultured skin-homing T lymphocytes using RT-PCR after 630 ± 3 nm LED irradiation was performed 8 times. T-cells can be classified into two groups depending on their cytokine profile²¹. Th-1 cells produce IL-2 and interferon- γ , whereas Th-2 cells produce IL-4 and IL-5. Th-1 cytokines lead predominantly to delayed-type hypersensitivity, while Th-2 cytokines induce the synthesis of IgE and lead to IgE-mediated allergy. The increase in IL-2 mRNA observed in the present study indicated an increase in activated Th-1 cells, and the increase in IL-4 mRNA indicated an increase of activated Th-2 cells. Both types of lymphocytes were activated by 630 ± 3 nm LED radiation, although the degree of activation seen in Th-2 cells was much more pronounced than in Th-1 cells (**Table 2**), findings that agree with the results of our previous laser study¹⁰.

An interesting finding was that morphological changes were minimal after 630 ± 3 nm LED phototherapy. Ultrastructurally, mild interstitial edema and slight fibroplastic changes (increases in the fibroblast count and in the number of vimentin granules and mitochondria in fibroblasts) were observed without dermal damage or acute inflammatory changes, which we observed after laser treatment in our previous study¹⁰. In previous studies by us and others, no amplification of cultured skin-homing T-lymphocytes has been observed in normal skin^{10,17,18}. However, the paucity of inflammation does not exclude the presence in skin treated with $630 \text{ nm} \pm 3 \text{ nm}$ LED of Th-2-type skin-homing T-cells, which were propagated in the culture system.

The increase in the IL-4 mRNA level and the slight histological changes suggest that clinical application of 630 ± 3 nm LED phototherapy is extremely promising.

In conclusion, these results suggest that 630 ± 3 nm LED phototherapy activates the immune system in normal skin. In patients with immunomodulated skin diseases, such as atopic dermatitis, where the function of the skin-homing T-cells has been altered^{9,17,22}, the induction of large amounts of skin-homing T-cells, particularly those of the Th-2 type, by 630 ± 3 nm LED phototherapy may help to improve the disease process as well as control the inflammatory process and accelerate wound healing. Further studies on this interesting aspect of visible red LED phototherapy are thus warranted.

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References

1. Mendez TM, Pinheiro AL, Pacheco MT, Nascimento PM, Ramalho LM: Dose and wavelength of laser light have influence on the repair of cutaneous

- wounds. *J Clin Laser Med Surg* 2004; 1: 19-25.
2. Rochkind S, Kogan G, Luger EG, et al: Molecular structure of the bony tissue after experimental trauma to the mandibular region followed by laser therapy. *Photomed Laser Surg* 2004; 22: 249-253.
 3. Neiburger EJ: Rapid healing of gingival incisions by the helium-neon diode laser. *J Mass Dent Soc* 1999; 48: 8-13.
 4. Woodruff LD, Bounkeo JM, Brannon WM, et al: The efficacy of laser therapy in wound repair: a meta-analysis of the literature. *Photomed Laser Surg* 2004; 22: 241-247.
 5. Albertini R, Aimbire FS, Correa FI, et al: Effects of different protocol doses of low power gallium-aluminum (Ga-Al-As) laser radiation (650 nm) on carrageenan induced rat paw oedema. *J Photochem Photobiol B* 2004; 74: 101-107.
 6. Whelan HT, Amits RL Jr, Buchman EV, et al: Effect of NASA light-emitting diode irradiation on wound healing. *J Clin Laser Med Surg* 2001; 19: 305-314.
 7. Whelan HT, Connelly JF, Hodgson BD, et al: NASA light-emitting diodes for the prevention of oral mucositis in pediatric bone marrow transplant patients. *J Clin Laser Med Surg* 2002; 20: 319-324.
 8. Whelan HT, Buchmann EV, Dhokalia A, et al: Effects of NASA light-emitting diode irradiation on molecular changes for wound healing in diabetic mice. *J Clin Laser Med Surg* 2003; 21: 67-74.
 9. Thestrup-Pedersen K: Atopic Dermatitis. In *Skin Immune System*, 2nd edit, (Bos JD, ed), pp 497-509, CRC Press, New York.
 10. Omi T, Kawana S, Sato S, et al: Cutaneous immunological activation elicited by a low-fluence pulsed dye laser. *Br J Dermatol* 2005; 153: 57-62.
 11. Kaltoft K, Perersen CB, Hansen BH, Lemonidis AS, Frydenberg J, Thestrup-Pedersen K: *In vitro* genetically aberrant T-cell clones with continuous growth are associated with atopic dermatitis. *Arch Dermatol Res* 1994; 287: 42-47.
 12. Kaltoft K, Hansen BH, Perersen CB, Pedersen S, Thestrup-Pedersen K: Common clonal chromosome aberrations in cytokine-dependent continuous human T-lymphocyte cell lines. *Cancer Genet Cytogenet* 1995; 85: 68-71.
 13. Omi T, Kawana S, Asano G, Honda M, Thestrup-Pedersen K, Hankins RW: Culture of skin-homing T-cells from lesional skin of atopic dermatitis patients using IL-2, IL-4. *J Pediat Dermatol* 2002; 21: 33-39.
 14. Taniguchi T, Matsui H, Fujita T, et al: Structure and expression on a cloned cDNA for human interleukin-2. *Nature* 1983; 302: 305-310.
 15. Nakazawa M, Sugi N, Kawaguchi H, Ishii N, Nakajima H, Minanii M: Predominance of type 2 cytokine-producing CD4+ and CD8+ cells in patients with atopic dermatitis. *J Allergy Clin Immunol* 1997; 99: 673-682.
 16. Kagi MK, Wuthrich B, Montano E, Barandun J, Blazer K, Walker C: Differential cytokine profiles in peripheral blood lymphocyte supernatants and skin biopsies from patients with different forms of atopic dermatitis, psoriasis and normal individuals. *Int Arch Allergy Immunol* 1994; 103: 332-340.
 17. Bang K, Lund M, Mogensen SC, Thestrup-Pedersen K: *In vitro* culture of skin-homing T lymphocytes from inflammatory skin diseases. *Exp Dermatol* 2005; 14: 389-395.
 18. Bang K, Lund M, Wu K, Mongensen SC, Thestrup-Pedersen K: CD4+ CD8+ (thymocyte-like) T lymphocytes present in blood and skin from patients with atopic dermatitis suggest immune dysregulations. *Br J Dermatol* 2001; 44: 1140-1147.
 19. Maeda A, Schwarz A, Kemebeck K, et al: Intravenous infusion of syngeneic apoptotic cells by photopheresis induces antigen-specific regulatory T cells. *J Immunol* 2005; 174: 5968-5976.
 20. Heshmati F: Mechanisms of action of extracorporeal photochemotherapy. *Transfus Apher Sci* 2003; 29: 61-70.
 21. Mosmann TR, Sas S: The expanding universe of T-cell subsets; Th-1, Th-2 and more. *Immunol Today* 1996; 17: 138.
 22. Akdis M, Simon HU, Weigl L, Kreyden O, Blaser K, Akdis CA: Skin homing (cutaneous lymphocyte-associated antigen-positive) CD8+ T Cells respond to superantigen and contribute to eosinophilia and IgE production in atopic dermatitis. *J Immunol* 1999; 163: 466-475.

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